

MerF is a mercury transport protein: different structures but a common mechanism for mercuric ion transporters?

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Abstract Mercury resistance determinants are widespread in Gram-negative bacteria, but vary in the number and identity of genes present. We have shown that the *merF* gene from plasmid pMER327/419 encodes a 8.7 kDa mercury transport protein, by determining in vivo mercury volatilisation when MerF is expressed in the presence of mercuric reductase. We have confirmed that MerC of Tn21 is also a mercuric ion transporter. We have been able to detect interaction of the periplasmic protein MerP only with the MerT transporter, and not with MerF or MerC. Hydropathy analysis led to the prediction of models for MerT, MerC and MerF having three, four and two transmembrane regions respectively. In all three cases one pair of cysteine residues is predicted to be within the inner membrane with a second pair of cysteine residues on the cytoplasmic face, and the second helix contains a proline and at least one charged residue. The mechanisms of mercuric ion transport may be similar in these transporters even though their structures in the membrane differ.

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Key words: MerT; MerC; MerF; Ion transport; Mercury resistance; Integral membrane protein; Protein structure

1. Introduction

Mercuric ion resistance in Gram-negative bacteria is widely distributed and is encoded by *mer* operons, usually located on transposons or plasmids (for recent reviews see [1–3]). Resistance is due to Hg(II) being taken up into the cell and delivered to the NADPH-dependent flavoenzyme mercuric reductase (E.C. 1.16.1.1). Mercuric reductase (MR, the *merA* gene product) catalyses the two-electron reduction of Hg(II) to the volatile low-toxicity Hg(0) [4]. Uptake of Hg(II) ions is required to confer resistance in vivo and is the rate-limiting step in resistance encoded by the *mer* determinants of Tn21 and Tn501 [5,6].

The *mer* operon of transposon Tn501 from *Pseudomonas aeruginosa* encodes two proteins implicated in mercuric ion transport, *merP* and *merT* [7,8]. That from Tn21 encodes an additional gene *merC*, located between *merP* and *merA* [9,10],

which has been suggested to encode a mercury transport protein [11], by analogy with the *merC* gene of *Thiobacillus ferrooxidans* [12,13]. Plasmid pMER327/419 from *Pseudomonas fluorescens* [14] also contains an open reading frame, designated *merF*, between *merP* and *merA*.

The mechanism of protein-mediated transport of mercuric ions across the bacterial membrane is not yet understood in any great detail. In Tn501 MerT two cysteines in the first transmembrane region are both required for Hg(II) transport and a second pair of cysteines, located on the cytoplasmic face of the membrane, are essential to maximal transport [8].

Here we have tested the hypothesis that MerF is a mercuric ion transport protein, investigated possible interactions of the known mercuric ion transporters with the periplasmic protein (MerP), and sought common features between the known mercuric transport proteins in order to develop further the model for the transport of Hg(II) across the cytoplasmic membrane.

2. Materials and methods

2.1. Molecular genetics

Plasmids used in this work are detailed in Table 1. DNA manipulations were carried out according to the methods outlined in [15] in *E. coli* TG2 (*supE*, *hsdΔ5*, *thi*, Δ *lac-proAB*, Δ *srl-recA*, 306::Tn10, F'[*traD36*, *proAB*⁺, *lacI*^q, *lacZΔM15*]) [16], except expression studies with pUC18/19-derived plasmids that were performed in *E. coli* CSH26Δ*recA* (Δ *lac-proAB*, *ara*, *thi*, Δ *recA*) [17]. DNA-modifying enzymes were purchased from Gibco-BRL, Paisley, UK. The *merT*, *merP* (from Tn501), *merF* (from pMER327/419) and *merC* (from Tn21) genes were cloned directly from other vectors, as outlined in Table 1, into the multiple cloning sites of either pUC18 or pUC19 vectors, in an orientation such that the expression of the *mer* gene(s) was from the *lac* promoter.

Differences in translation initiation of the *merC* gene and *merF* gene were minimised in volatilisation assays by cloning PCR products in place of *merT* in Tn501 and maintaining the *merT* translation initiation region. Amplification of *merC* used Vent[®] DNA polymerase (New England Biolabs, Herts, UK) with primers shown in Table 2 for 24 cycles of: 96°C, 40 s; 55°C (cycles 1–3) or 80°C (cycles 4–24), 60 s; 72°C, 120 s. For *merF* amplification used different primers (Table 2) and 70°C was used instead of 80°C for cycles 4–24. The products were cloned into the *LspI*-*BglII* linker which replaces the *merT* gene in pBR*merBS2ΔT* (Table 1), to produce pBR-C and pBR-F. Expression of *merC* or *merF* was regulated by MerR and co-expressed with *merP*, *A* and *D*.

2.2. Localisation of *merT*, *merC* and *merF* gene products

A modification of the 'maxicell' method of [18] was used to confirm the expression of the MerC, MerF and MerT proteins and to determine their subcellular location. Cultures of *E. coli* CSH26Δ*recA* (pCAH52) containing pUC18/19-derived plasmids were grown to mid-log phase at 37°C in M9 medium [17], UV-irradiated and then incubated (37°C, 1 h) in the dark before adding 1-cycloserine (Sigma Chemical Co., UK) to 100 μM. Incubation was continued overnight, cells were harvested, washed and resuspended in M9 salts containing

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Table 1
Plasmids used in this study

Plasmid	Relevant features ^a or description	Reference or source
pUC18	Ap ^R ; multiple cloning site adjacent to <i>lac</i> promoter	[30]
pUC19		
pACAH52	pACYC184-derived plasmid constitutively expressing <i>merA</i> from the CAT promoter	[5]
pBR322	Ap ^R	[31]
pBR _{mer} BS2	Ap ^R ; 4.3 kb <i>EcoRI</i> / <i>NruI</i> fragment containing <i>merRTPAD</i> genes of Tn501 subcloned from pUB3466BS2 [8] into the <i>EcoRI</i> / <i>NruI</i> sites of pBR322	This work
pBR _{mer} BS2ΔP	Ap ^R ; <i>Bgl</i> II/ <i>Bam</i> HI deletion of <i>merP</i> from pBR _{mer} BS2	This work
pBR _{mer} BS2ΔT	Ap ^R ; pBR _{mer} BS2 cut with <i>Lsp</i> I and <i>Bgl</i> II to remove the <i>merT</i> gene and replaced with a <i>Lsp</i> I/ <i>Bgl</i> II linker	This work
pBR-C	Ap ^R ; <i>merC</i> from pUC19C cloned into the <i>Lsp</i> I/ <i>Bgl</i> II sites of pBR _{mer} BS2ΔT vector; giving <i>merRCPAD</i>	This work
pBR-CΔP	Ap ^R ; <i>Bgl</i> II/ <i>Bam</i> HI deletion of <i>merP</i> from pBR-C	This work
pBR-F	Ap ^R ; <i>merF</i> from pUC18F cloned into the <i>Lsp</i> I/ <i>Bgl</i> II sites of pBR _{mer} BS2ΔT; giving <i>merRFPAD</i>	This work
pBR-FΔP	Ap ^R ; <i>Bgl</i> II/ <i>Bam</i> HI deletion of <i>merP</i> from pBR-F	This work
pUC18TP	<i>merT</i> and <i>merP</i> cloned from pT7ScmerTP [32] into the <i>EcoRI</i> / <i>Bam</i> HI sites of pUC18	This work
pUC18T	<i>Bgl</i> II/ <i>Bam</i> HI deletion of <i>merP</i> from pUC18TP	This work
pUC19C	1652 bp <i>Nhe</i> I/ <i>Eco</i> RI DNA fragment containing <i>merC</i> cloned from pDU1205 [33] into pUC13	This work
	<i>Xba</i> I/ <i>Eco</i> RI and subcloned as a <i>Pst</i> I/ <i>Eco</i> RI fragment into the <i>Pst</i> I/ <i>Eco</i> RI sites of pUC19	
pUC18F	380 bp <i>Kpn</i> I/ <i>Xmn</i> I fragment containing <i>merF</i> subcloned from pDGJ118 [34] into the <i>Kpn</i> I/ <i>Hinc</i> II sites in pUC18	This work

^aAp, ampicillin; CAT, chloramphenicol acetyl transferase.

10.5% (w/v) cysteine-free assay medium (Difco Laboratories, Detroit, USA) and 100 μM 1-cycloserine, then ³⁵S-labelled methionine/cysteine Translabel (2 μCi; ICN, UK) was added. Gene expression was induced for 1 h by addition of IPTG to 1 mM. Cells were harvested, lysed by sonication and unlysed cells were removed by low speed centrifugation. Crude membrane fractions were isolated by centrifugation (100 000 × g, 1.5 h) and resuspended in 100 mM Tris-Cl, pH 6.8, 10% glycerol, 0.4% SDS and 1% 2-mercaptoethanol for analysis. Polyacrylamide slab gel electrophoresis was performed according to [19]. The gel was dried and a digital image of labelled material was obtained using a Molecular Dynamics phosphorimager system. A control periplasmic fraction was obtained from labelled cells by the MgSO₄ method described in [20].

2.3. Mercury volatilisation assays

Mercury volatilisation assays were performed using a method adapted from [21]. Overnight shake cultures of *E. coli* TG2 (pBR_{mer}) or related cells in M9 broth [17] at 37°C were diluted into fresh M9 medium and grown to mid-log phase. Expression of the *mer* genes was induced with 1 μg ml⁻¹ HgCl₂ for 1 h, cells were harvested and a constant number of cells (approximately 2 × 10⁸) were resuspended in 100 μl of M9 medium supplemented with 5 μM HgCl₂ and 1 mM IPTG.

The cell suspension was transferred to 5 ml of the same buffer containing ²⁰³Hg (1 μCi ml⁻¹; Amersham, UK) and incubated with shaking at 37°C. Timed 100 μl aliquots were transferred to scintillation fluid (5 ml; Optiphase Hisafe, Wallac, Finland) and the amount of ²⁰³Hg was quantified by scintillation counting.

2.4. Computer analysis of protein sequences

DNA and peptide sequence data were analysed using the GCG suite of programs (Version 10.0, Genetics Computer Group, Madison, WI, USA). Secondary structure motifs were identified from the hydrophathy algorithms PEPTIDESTRUCTURE, from the GCG suite, and the transmembrane helix identification program DAS [22].

3. Results

3.1. Localisation of *merT*, *merC* and *merF* gene products

Membrane fractions from *E. coli* CSH26Δ*recA* cells expressing the *merTP*, *merT*, *merC*, or *merF* genes in pUC18/19 contained radiolabelled protein of the molecular masses expected from their primary structures (Fig. 1), except MerT which runs at a higher apparent *M_r*, as found previously

[8,23–25]. The MerC and MerF proteins (tracks 4 and 5, Fig. 1) have *M_r* values of 15 000 and 8700 respectively. A periplasmic fraction prepared from the same cells showed no proteins of equivalent size (data not shown) indicating that these are not transient periplasmic proteins. MerF was less well expressed than the other proteins and the longer incubation with ³⁵S gives additional labelled proteins from MerF-containing cells. The reasons for the lower apparent expression of MerF (e.g. expression, membrane incorporation or protein turnover) were not addressed in the present study. MerC and MerF showed better expression when expressed from the MerT translation initiation region than when expressed using their own initiation sequences (data not shown).

3.2. Mercury volatilisation

E. coli TG2 (pBR_{mer}) cells containing the *merT*, *merTP*, *merC*, or *merF* genes cloned in pBR_{mer} showed an increase in mercury volatilisation compared to cells containing pBR_{mer}ΔT (Fig. 2). Volatilisation was lower than in an equivalent amount of sonicated cell-free extract, indicating that in each case transport was rate-limiting for resistance. The highest rate of cellular mercury volatilisation was produced when both MerT and MerP were expressed, with MerT alone giving approximately 20% less volatilisation. MerC showed greater

Table 2
PCR primers for amplification of *merC* and *merF*

Primer	Sequence ^a
<i>merC</i> (SDmerT)	5′-TTCGAA AGGACA AGCGCATGGGACTGATGACACGC-3′
<i>merC</i> (rev)	5′-AGATCTCCGATCACAAGCGCTTGGC-3′
<i>merF</i> (SDmerT)	5′-TTCGAA AGGACA AGCGCATGAAAGACCCGAGAC-3′
<i>merF</i> (rev)	5′-AGATCTCCGATCATTCTTTTACTCCATTGATTGG-3′

^aRestriction endonuclease sites are given in italics (*Lsp*I, TTCGAA; *Bgl*II, AGATCT), the Shine-Dalgarno sequence of the MerT translation initiation region is in bold and the start codon is underlined.

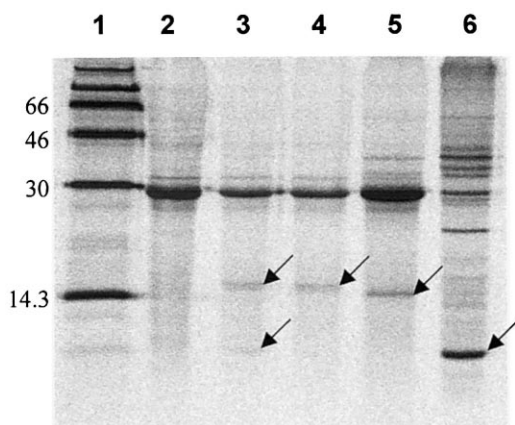


Fig. 1. SDS-polyacrylamide gel electrophoresis of ^{35}S -labelled proteins from membrane fractions. The tracks are (1) ^{14}C molecular weight markers (Amersham, UK) with sizes in kDa; membrane fractions from *E. coli* CSH26 Δ recA (pACAH52) cells containing: (2) pUC18; (3) pUC18TP; (4) pUC18T; (5) pUC19C; (6) pUC18F. Arrows mark the proteins MerT (and a trace of MerP), MerC and MerF in tracks 3–6.

volatilisation than MerF, but neither MerC nor MerF showed any increase in the rate of mercury volatilisation in the presence of MerP. Interestingly, the expression of MerP and MerA in the absence of MerT, MerC or MerF gave a higher level of volatilisation than the strain expressing MerA alone.

3.3. Analysis of protein sequences of MerT, MerC and MerF

Hydropathy prediction programs indicated that MerT has three membrane-spanning regions, MerC has four and MerF has two (Fig. 3A); the first region in each case containing two closely spaced cysteine residues and a nearby distal proline residue (Fig. 3B). The second hydrophobic region contains one or more charged residues, with an adjacent proline (Fig. 3B). Each protein also contains an additional pair of cysteine residues predicted to be on the cytoplasmic face of the membrane, as they are found in regions containing positive charges (Fig. 3B). In MerT this is located between transmembrane regions II and III and in MerC and MerF this is close to the carboxyl-terminal (Fig. 3A).

4. Discussion

4.1. Identification of mercury transport genes

The data reported here indicate that MerF and MerC are bona fide mercuric ion transport proteins which carry Hg(II) to mercuric reductase. In vivo expression of MerF in the presence of reductase gives increased volatilisation over an isogenic strain lacking the *merF* gene, although the phenotypic effects are less pronounced than for MerC, or for MerT and MerTP. Although the role of Tn21 MerC has previously been deduced by comparison with the MerC of *T. ferro-oxidans* and uptake has been demonstrated [12,13], this is the first time its transport rather than binding properties [26,27] have been demonstrated. Our data on MerT and MerP agree with earlier observations [8].

The significance of differences in quantitative effects of expression of MerT (\pm MerP), MerC and MerF is difficult to determine. The expression of the transport proteins was likely to be different in pUC vectors, as their own translation initiation regions were used, and some evidence for this came

from 'maxicell' labelling experiments (Fig. 1) in which the MerF product was always present in much lower amounts than MerT or MerC. When the transport proteins were expressed in pBRmer derivatives, MerT, MerC and MerF were all expressed from the translation initiation region of MerT up to the initiation codon, and are apparently produced in similar amounts (data not shown), but they may affect the downstream expression of the mercuric reductase in this case. Such expression differences are difficult to control, but do not affect the qualitative observations.

We have previously shown that MerP and MerT together are more efficient in mercuric ion uptake and volatilisation than MerT alone [8]; however, we show here that MerP makes no apparent difference to the rate of volatilisation in the presence of MerC or MerF, indicating that neither of these transport proteins interacts with MerP in vivo. Transport is rate-limiting for volatilisation, as shown using a cell-free extract. The volatilisation activity therefore represents the rate of mercuric ion transport of the different *mer* proteins.

Expression of MerP and mercuric reductase (in the absence of MerT, MerC or MerF) gives a slightly higher rate of volatilisation than the expression of mercuric reductase alone (Fig. 2, pBRmerBS2 Δ T and pBRmerBS2 Δ ATP). The reasons for this are not clear, but MerP may increase the local concentration of mercuric ions in the periplasmic compartment and allow increased transmembrane passage of partially covalent Hg(II) compound. Any effects must be subtle as there is no detectable effect of MerP in the presence of MerC or MerF, and can be differentiated from the pronounced effect of MerP on transport by MerT. Unlike MerT, MerC or MerF, MerP does not cause increased sensitivity to HgCl₂ in the absence of mercuric reductase, or increased resistance in its presence, when measured by minimum inhibitory concentration (data not shown), indicating that MerP does not act to transport Hg(II) directly.

4.2. A unitary model for mercuric ion transport

The hydropathy prediction programs indicated that the numbers of membrane-spanning regions in the three proteins are different: MerT-3; MerC-4; MerF-2 (Fig. 3A). Each pro-

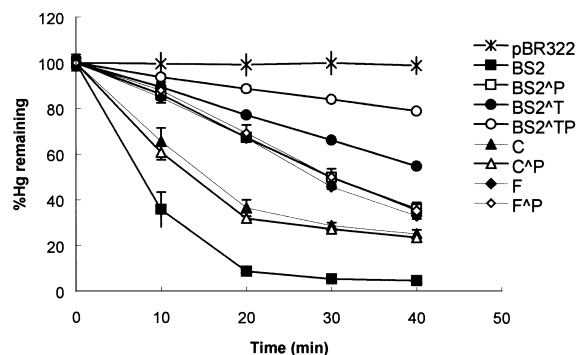


Fig. 2. Assay showing the volatilisation of Hg(II) from cultures of *E. coli* TG2 cells containing pBRmer and its derivatives. The amount of ^{203}Hg remaining in the assay buffer at each time point was subtracted from the initial value and expressed as a percentage of initial ^{203}Hg . Each sample was assayed in triplicate and all assays were done in parallel. The symbols are: cells containing pBRmerBS2 (■), pBRmerBS2 Δ P (□), pBRmerBS2 Δ T (●), pBRmerBS2 Δ ATP (○), pBR-C (▲), pBR-C Δ P (△), pBR-F (◆), pBR-F Δ P (◇) and pBR322 (*).

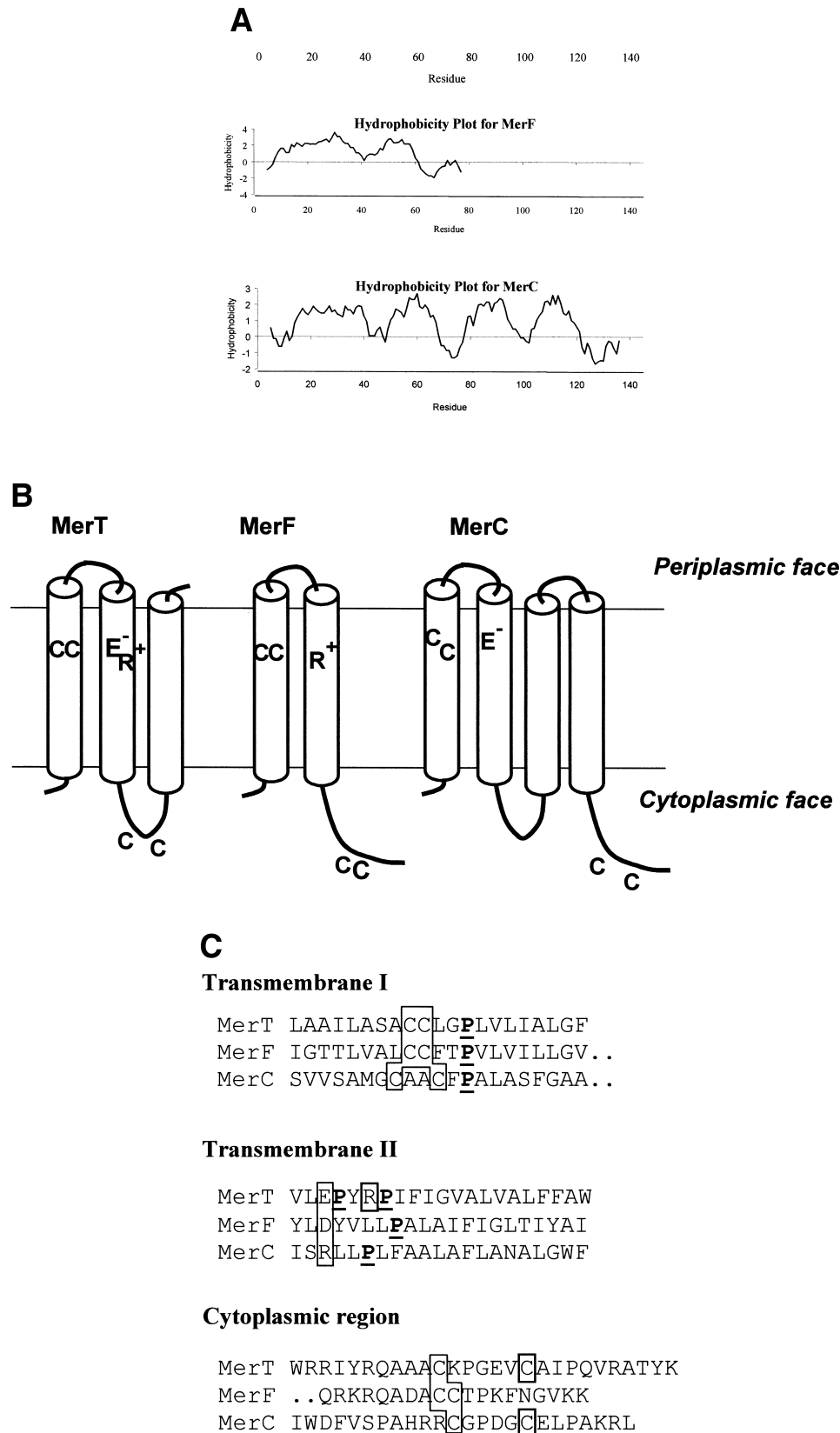


Fig. 3. The conserved features of the MerT, MerC and MerF proteins and alignment of key elements. A: Output of PEPTIDESCAN hydropathy plots. B: Topological alignment of all amino acids separated according to the predicted hydrophobic (membrane-spanning) elements. C: Structural elements of the transporters aligned according to the predicted topology: the cysteines in the first transmembrane region and in the cytoplasmic regions of MerT, MerF and MerC are boxed, as are the charged residues in the second transmembrane region; prolines in the first and second transmembrane region are underlined and in bold.

tein has a cysteine pair located in the first transmembrane region: Cys-Cys in MerT and MerF, and Cys-Ala-Ala-Cys in MerC (Fig. 3B). Given the structure of an α -helix, the two types of motif may not represent significant chemical differences. There is a clear evidence from mutagenesis studies on MerT [8] and MerC (this work and [26]) that these cysteine residues are required for mercuric ion transport. We predict that they are also required in MerF.

Proline residues are conserved on the carboxy-terminal side of this cysteine motif and may also be of structural importance, perhaps serving to expose the Cys residues to solvent, as may the proline residue(s) in the second transmembrane regions (Fig. 3B). In all three proteins a charged residue is located to the periplasmic side of this second proline residue. We propose that a charged or polar residue within this region is important for transport. This has been confirmed for MerT (Leang, Brown and Wilson, in preparation).

The other major structurally equivalent region identified for each protein is a cysteine pair on the cytoplasmic face of the protein, shown in Fig. 3. In all three proteins this region contains a high proportion of charged residues, including positively charged amino acids, and this region may be involved in the transfer of mercuric ions to the N-terminal domain in MR [28,29], as mutation of these cysteine residues in MerT causes a reduction in mercuric ion transport [8].

From these analyses we conclude that MerT, MerC and MerF may function by equivalent mechanisms, with mercury-binding cysteines within the transmembrane helix, exposed to the periplasmic compartment, and functional cysteines on the cytoplasmic face of the inner membrane. The effect of the different number of transmembrane regions is not known; they may affect the stability of the transport proteins in the membrane or the formation of an oligomeric complex. The MerF monomer is one of the smallest gene products known which constitutes a transport system.

Although our data show that the *merT*, *merC* and *merF* genes all encode mercuric ion transport genes and we hypothesise that their mechanisms of action are similar, we have already shown differences in their interactions with MerP and cannot yet address the reasons why the *merC* and *merF* genes are found in some operons but not others. It is entirely possible that the K_m for Hg(II) is different for each of the transporters, permitting efficient mercuric ion resistance at different Hg(II) concentrations. Testing this hypothesis will require the separate overproduction and partial purification of each of the transport proteins.

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References

- [1] Hobman, J.L. and Brown, N.L. (1997) in: Mercury and its Effects on Environment and Biology (Sigel, A. and Sigel, H., Eds.), Metal Ions in Biol. Systems Vol. 34, pp. 527–568, Marcel Dekker, New York.
- [2] Osborn, A.M., Bruce, K.D., Strike, P. and Ritchie, D.A. (1997) FEMS Microbiol. Rev. 19, 239–262.
- [3] Silver, S. and Phung, L.T. (1996) Annu. Rev. Microbiol. 50, 753–789.
- [4] Fox, B.S. (1982) J. Biol. Chem. 257, 2498–2503.
- [5] Lund, P.A. and Brown, N.L. (1987) Gene 52, 207–214.
- [6] Nakahara, H., Silver, S., Miki, T. and Rownd, R.H. (1979) J. Bacteriol. 140, 161–166.
- [7] Misra, T.K., Brown, N.L., Fritzinger, D.C., Pridmore, R.D., Barnes, W.M., Haberstroh, L. and Silver, S. (1984) Proc. Natl. Acad. Sci. USA 81, 5975–5979.
- [8] Morby, A.P., Hobman, J.L. and Brown, N.L. (1995) Mol. Microbiol. 17, 25–35.
- [9] Barrineau, P. and Summers, A.O. (1983) Gene 25, 209–222.
- [10] Misra, T.K., Brown, N.L., Haberstroh, L., Schmidt, A., Godette, D. and Silver, S. (1985) Gene 34, 253–262.
- [11] Hamlett, N.V., Landale, E.C., Davis, B.H. and Summers, A.O. (1992) J. Bacteriol. 174, 6377–6385.
- [12] Inoue, C., Kusano, T. and Silver, S. (1996) Biosci. Biotech. Biochem. 60, 1289–1292.
- [13] Kusano, T., Ji, G., Inoue, C. and Silver, S. (1990) J. Bacteriol. 172, 2688–2692.
- [14] Hobman, J., Kholodii, G., Nikiforov, V., Ritchie, D.A., Strike, P. and Yurieva, O. (1994) Gene 146, 73–78.
- [15] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- [16] Gibson, T.J. (1984) PhD Thesis, Cambridge University, Cambridge.
- [17] Miller, J.H. (1972) Experiments in Microbial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- [18] Sancar, A., Hack, A.M. and Rupp, W.D. (1979) J. Bacteriol. 137, 692–693.
- [19] Laemmli, U.K. (1970) Nature 227, 680–685.
- [20] Asubel, F.M. (1989) Current Protocols in Molecular Biology, Wiley, New York.
- [21] Ji, G., Salzberg, S.P. and Silver, S. (1989) Appl. Environ. Microbiol. 55, 523–525.
- [22] Cserzo, M., Wallin, E., Simon, I., von Heijne, G. and Elofsson, A. (1997) Protein Eng. 10, 673–676.
- [23] Jackson, W.J. and Summers, A.O. (1982) J. Bacteriol. 149, 479–487.
- [24] Jackson, W.J. and Summers, A.O. (1982) J. Bacteriol. 151, 962–970.
- [25] Ni'Bhriain, N. (1986) Gene 42, 323–330.
- [26] Sahlman, L., Häggblöf, E.-M. and Powlowski, J. (1999) Biochem. Biophys. Res. Commun. 255, 307–311.
- [27] Sahlman, L., Wong, W. and Powlowski, J. (1997) J. Biol. Chem. 272, 29518–29526.
- [28] Brown, N.L. (1985) Trends Biochem. Sci. 10, 400–403.
- [29] Summers, A.O. (1986) Annu. Rev. Microbiol. 40, 607–634.
- [30] Vieira, J. and Messing, J. (1982) Gene 19, 259–268.
- [31] Bolivar, F. (1978) Gene 4, 121–136.
- [32] Hobman, J.L. and Brown, N.L. (1996) Mol. Gen. Genet. 250, 129–134.
- [33] Foster, T.J. and Brown, N.L. (1985) J. Bacteriol. 163, 1153–1157.
- [34] Hobman, J.L. (1992) PhD Thesis, The University of Liverpool, Liverpool.